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14,15-Dihydroxy-eicosa-5(*Z*)-enoic Acid Selectively Inhibits 14,15-Epoxyeicosatrienoic Acid-Induced Relaxations in Bovine Coronary Arteries

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ABSTRACT

Cytochrome P-450 epoxygenases metabolize arachidonic acid (AA) to epoxyeicosatrienoic acids (EETs). EETs relax vascular smooth muscle by membrane hyperpolarization. 14,15-Epoxyeicosa-5(*Z*)-enoic acid (14,15-EE5ZE) antagonizes many vascular actions of EETs. EETs are converted to the corresponding dihydroxyeicosatrienoic acids by soluble epoxide hydrolase (sEH). sEH activity in the bovine arterial endothelium and smooth muscle regulates endogenous EETs. This study examined sEH metabolism of 14,15-EE5ZE to 14,15-dihydroxy-eicosa-5(*Z*)-enoic acid (14,15-DHE5ZE) and the resultant consequences on EET relaxations of bovine coronary arteries (BCAs). BCAs converted 14,15-EE5ZE to 14,15-DHE5ZE. This conversion was blocked by the sEH inhibitor 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA). 14,15-EET relaxations (maximal relaxation, 83.4 $\pm$ 4.5%) were inhibited by 14,15-DHE5ZE (10 μM; maximal relaxation, 36.1 $\pm$ 9.0%; $p < 0.001$). In sharp contrast with 14,15-EE5ZE, 14,15-DHE5ZE is a 14,15-EET-selective inhibitor and did not inhibit 5,6-, 8,9-, or 11,12-EET relaxations. 14,15-EET and 11,12-EET relaxations were similar in the presence and absence of AUDA (1 μM). 14,15-EE5ZE inhibited 14,15-EET relaxations to a similar extent with and without AUDA pretreatment. However, 14,15-EE5ZE inhibited 11,12-EET relaxations to a greater extent with than without AUDA pretreatment. These observations indicate that sEH converts 14,15-EE5ZE to 14,15-DHE5ZE, and this alteration influences antagonist selectivity against EET-regioisomers. 14,15-DHE5ZE inhibited endothelium-dependent relaxations to AA but not endothelium-independent relaxations to sodium nitroprusside. A series of sEH-resistant ether analogs of 14,15-EE5ZE was developed, and analogs with agonist and antagonist properties were identified. The present study indicates that conversion of 14,15-EE5ZE to 14,15-DHE5ZE produces a 14,15-EET-selective antagonist that will be a useful pharmacological tool to identify EET receptor(s) and EET function in the cardiovascular system.

Introduction

Endothelial cells metabolize arachidonic acid (AA) through the cyclooxygenase, lipoxygenase, and cytochrome P450 (P450) epoxygenase pathways (Rosolowsky and Campbell, 1993, 1996). The P450 pathway produces four regioisomeric epoxyeicosatrienoic acids (EETs): 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET. Early studies have revealed diverse physiological functions of EETs, including dilatation of coronary, renal, and cerebral arteries; smooth muscle, endothelial, and epithelial cell proliferation; and anti-inflammatory effects in vascular tissue (Node et al., 1999; Campbell and Falck, 2007; Larsen et al., 2007; Spector and Norris, 2007). EETs function as endothelium-derived hyperpolarizing factors in the coronary circulation and cause vascular smooth muscle relaxation by activating large-conductance, Ca^{2+}-activated K^+ channels and membrane hyperpolarization (Campbell et al., 1996; Fisslthaler et al., 1999; Campbell and Falck, 2007). These vascular

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ABBREVIATIONS: AA, arachidonic acid; P450, cytochrome P450; EET, epoxyeicosatrienoic acid; 14,15-EE5ZE, 14,15-epoxyeicosa-5(*Z*)-enoic acid; DHET, dihydroxyeicosatrienoic acid; sEH, soluble epoxide hydrolase; BCA, bovine coronary artery; 14,15-DHE5ZE, 14,15-dihydroxyeicosa-5(*Z*)-enoic acid; 13-HTDA, 13-(heptyloxy)tridec-5(*Z*)-enoic acid; 14-HTDA, 14-(hexyloxy)tetradec-5(*Z*)-enoic acid; 15-PPDA, 15-(pentyloxy)pentadec-5(*Z*)-enoic acid; 14-HTD-8Z-A, 14-(hexyloxy)tetradec-8*Z*-enoic acid; U46619, 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$; SNP, sodium nitroprusside; TBS, Tris-buffered saline; DAPI, 4′,6′-diamino-2-phenylindole; AUDA, 12-(3-adamantan-1-yl-ureido)-dodecanoic acid; LC/ESI-MS, liquid chromatography-electrospray ionization-mass spectrometry; 5,6-EET-Me, methyl ester analog of 5,6-EET.

effects of EETs are selectively blocked by 14,15-epoxyeicosa-5(*Z*)-enoic acid (14,15-EE5ZE) (Gauthier et al., 2002a). EETs are rapidly converted to the corresponding dihydroxyeicosatrienoic acids (DHETs) in vascular cells by soluble epoxide hydrolase (sEH) (Spector and Norris, 2007). Vascular relaxation by the DHETs is variable, but they are generally less active than the EETs. 5,6-EET relaxes the rat tail artery; however, 5,6-DHET is without effect (Carroll et al., 1987). In contrast, 11,12-DHET is equipotent to 11,12-EET, producing relaxation in porcine coronary arteries (Weintraub et al., 1997; Oltman et al., 1998). 14,15-DHET induces relaxation of the bovine coronary artery (BCA) but is 5-fold less potent than 14,15-EET (Campbell et al., 2002). These studies indicate that metabolism of EETs plays an important role in regulating their biological activity.

sEH is a cytosolic enzyme that is widely distributed in mammalian tissues (Fretland and Omiecinski, 2000). sEH activity has been detected in arterial endothelial and smooth muscle cells (Fang et al., 1996, 2001; Campbell et al., 2002). Selective inhibition or deletion of sEH decreases blood pressure in rodents (Sinal et al., 2000; Imig et al., 2002; Imig and Hammock, 2009). Growing evidence indicates the utility of sEH inhibition in the prevention of cerebral ischemia, myocardial infarction, and hypertension (Gross et al., 2008; Imig and Hammock, 2009). 14,15-EE5ZE, a selective antagonist of EETs and structural analog of 14,15-EET, is a potential substrate for sEH, being converted to corresponding 14,15-dihydroxyeicosa-5(*Z*)-enoic acid (14,15-DHE5ZE). No studies have been conducted on the metabolism of 14,15-EE5ZE by sEH or the effect of 14,15-DHE5ZE on the actions of the EETs.

In the current work, we studied the expression of sEH protein and its role in metabolism of 14,15-EE5ZE to 14,15-DHE5ZE in BCAs. Furthermore, we characterized the inhibitory effect of 14,15-DHE5ZE against EET-induced relaxation. A series of sEH-resistant ether analogs of 14,15-EE5ZE was synthesized and tested for agonist and antagonist properties. The present studies indicate that BCAs and endothelial cells metabolize 14,15-EE5ZE to 14,15-DHE5ZE. 14,15-DHE5ZE inhibits 14,15-EET-induced relaxation but not the other EET regioisomers. Moreover, as a 14,15-EET-selective antagonist, 14,15-DHE5ZE may be useful in determining the physiological role of endogenous 14,15-EET.

Materials and Methods

Synthesis of EET Analogs. The EET analogs 14,15-EE5ZE, 14,15-DHE5ZE, 13-(heptyloxy)tridec-5(*Z*)-enoic acid (13-HTDA), 14-(hexyloxy)tetradec-5(*Z*)-enoic acid (14-HTDA), 15-(pentyloxy)pentadec-5(*Z*)-enoic acid (15-PPDA), 20-hydroxy-14-HTDA, and 14-(hexyloxy)tetradec-8*Z*-enoic acid (14-HTD-8Z-A) were synthesized as described previously (Falck et al., 1990, 2003).

Vascular Reactivity Studies. Measurements of isometric tone in BCA rings were conducted as described previously (Campbell et al., 1996; Gauthier et al., 2002a). Fresh bovine hearts were obtained from a local slaughterhouse. Sections of the left anterior descending coronary artery were dissected, cleaned, and cut into 1.5- to 2.0-mm diameter rings (3 mm length). The arterial rings were suspended in a tissue bath containing a Krebs bicarbonate buffer equilibrated with 95% O_2/5% CO_2 and maintained at 37°C. Tension was measured using a model FT-03C force transducer (Grass Instruments, Quincy, MA), ETH-400 bridge amplifier, and MacLab 8e A/D converter with MacLab software (ADInstruments, Colorado Springs, CO) and Macintosh computer. The arterial rings were slowly stretched to a basal tension of 3.5 g and equilibrated for 1.5 h. KCl (40–60 mM) was repeatedly added and rinsed until reproducible stable contractions were observed. The thromboxane mimetic 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619; 20 nM) was added to increase basal tension to approximately 50 to 75% of maximal KCl contraction. Vessels were pretreated for 5 min with vehicle, 14,15-EE5ZE (10 μM), 14,15-DHE5ZE (10 μM), or 13-HTDA (10 μM). Relaxation responses to cumulative additions of the EETs (10^{-9}-10^{-5} M), AA (10^{-8}-10^{-5} M), or sodium nitroprusside (SNP; 10^{-9}-10^{-5} M) were recorded. Basal tension represents tension before the addition of U46619. Results are expressed as percentage relaxation of the U46619-treated rings; 100% relaxation represents basal tension.

Western Immunoblotting. BCA rings were prepared as described under *Vascular Reactivity Studies*, and bovine coronary endothelial cells and smooth muscle cells were cultured as described previously (Campbell et al., 2002). Coronary artery lysates (25 μg) and recombinant human sEH protein (provided by Dr. Bruce Hammock, University of California, Davis, CA) were loaded in separate lanes and resolved by SDS-polyacrylamide gel electrophoresis using a 10% resolving gel and 4% stacking gel. Proteins were then transferred to nitrocellulose membranes. Nonspecific binding was blocked by incubation of the membrane with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (20 mM Tris base, 50 mM NaCl, and 10% Tween 20, pH 7.8) for 1 h at room temperature. Rabbit serum against human sEH was used (1:5000 dilution; provided by Dr. Bruce Hammock) in 5% milk-TBS buffer (20 mM Tris base and 50 mM NaCl, pH 7.8), and it was incubated for 1 h at room temperature. The membrane was rinsed with Tris-buffered saline containing 0.1% Tween 20 and TBS buffer. Goat antirabbit IgG conjugated to horseradish peroxidase (1:5000; Zymed Laboratories, South San Francisco, CA) was added as a secondary antibody in 5% milk-TBS buffer and incubated for 1 h at room temperature. Immunoreactive bands were visualized using Super-Signal Chemiluminescent Substrate (Pierce Chemical, Rockford, IL) and detected on Kodak BioMax ML film (Carestream Health, Rochester, NY).

Immunohistochemistry. BCAs (i.d. = 500–800) were sectioned and prepared as described previously (Gauthier et al., 2002b). Prepared sections were incubated for 2 h at room temperature with the sEH antibody (1:200 in 1% bovine serum albumin in phosphate-buffered saline). The slides were rinsed and incubated with antirabbit secondary antibody (1:1000; Alexa Fluor 594; Invitrogen, Carlsbad, CA) for 1 h at room temperature. After final rinse, the sections were covered with mounting media (Vectashield; Vector Laboratories, Burlingame, CA) containing 4′,6′-diamino-2-phenylindole·2HCl (DAPI; 1.5 μg/ml). To control for nonspecific fluorescence, sections were incubated with no antibodies or secondary antibody alone. Nomarski and fluorescent images were captured with a 20× magnification using a Nikon (Melville, NY) Eclipse E600 microscope and Spot Advanced software.

Metabolism of 14,15-EE5ZE and 13-HTDA by BCAs. BCAs were prepared as described under *Vascular Reactivity Studies* (Rosolowsky and Campbell, 1993; Yang et al., 2005). 14,15-EE5ZE or 13-HTDA (1 μM) was incubated for 10 or 30 min at 37°C in HEPES buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 6 mM glucose, pH 7.4) in the presence and absence of coronary artery rings. The 14,15-EE5ZE metabolism was repeated in the presence and absence of the sEH inhibitor 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA; 10^{-6} M; provided by Dr. Bruce Hammock). Epoxide hydrolase activity was expressed as the conversion of 14,15-EE5ZE to 14,15-DHE5ZE.

After the incubation, the samples were subjected to solid-phase extraction using C_{18} Bond Elut columns. The samples were dried under a stream of nitrogen and analyzed by liquid chromatography-electrospray ionization-mass spectrometry (LC/ESI-MS; 1100 LC/MSD, SL model; Agilent, Palo Alto, CA) as described previously (Nithipatikom et al., 2001; Yang et al., 2005). 14,15-EE5ZE, 14,15-DHE5ZE, and 13-HTDA were measured in the selected ion monitoring mode by detecting the M-H ions (molecular mass minus a hydro-

gen ion) of *m/z* 323, *m/z* 341, and *m/z* 325, respectively. In parallel analyses, the migration times of known standards (14,15-EE5ZE, 14,15-DHE5ZE, and 13-HTDA) were determined.

Statistical Analysis. The data are expressed as means ± S.E.M. Statistical analysis was performed by a one-way analysis of variance followed by the Student-Newman-Keuls multiple comparison test when significant differences were present. $P < 0.05$ was considered statistically significant.

Results

Expression of sEH in BCAs. Western immunoblotting with a specific sEH antibody detected an immunoreactive band (~60 kD) in BCA homogenates (Fig. 1A). Control lanes were loaded with 3 and 15 ng of human recombinant sEH. In BCA sections, fluorescent labeling with the sEH antibody resulted in a strong red fluorescent signal in the endothelium with a weaker staining of the smooth muscle (Fig. 1B, sEH). Arrows in Fig. 1 indicate the endothelial layer. Blue DAPI staining in Fig. 1 indicates the location of the nuclei. In sections prepared with the secondary antibody alone (Fig. 1B, control) or without antibodies (not shown), only weak staining was observed. Corresponding Nomarski images (Fig. 1B) are also shown. These results suggest that the endothelium is the primary cellular location of sEH activity in BCAs.

Metabolism of 14,15-EE5ZE and 13-HTDA in BCAs. To determine whether 14,15-EE5ZE and its ether analog 13-HTDA are substrates for sEH and metabolized by the BCA, segments of arteries were incubated with 14,15-EE5ZE and its conversion to 14,15-DHE5ZE was measured by LC/ESI-MS. As shown in Fig. 2A, no conversion of 14,15-EE5ZE to 14,15-DHE5ZE occurs in the absence of arteries. However, incubation of arteries with 14,15-EE5ZE resulted in a time-dependent increase in 14,15-DHE5ZE formation. The synthesis of 14,15-DHE5ZE was completely blocked with pretreatment with the sEH inhibitor AUDA (Fig. 2B). These data indicate that sEH is responsible for the metabolism of 14,15-EE5ZE to 14,15-DHE5ZE in coronary arteries. When arteries were incubated with 13-HTDA, which contains an ether rather than an epoxy group, no metabolism was observed (Fig. 2C). The amount of 13-HTDA was the same in the presence or absence of arteries and was unchanged with time of incubation. These data suggest that ether analogs of 14,15-EE5ZE such as 13-HTDA are resistant to the metabolism by sEH.

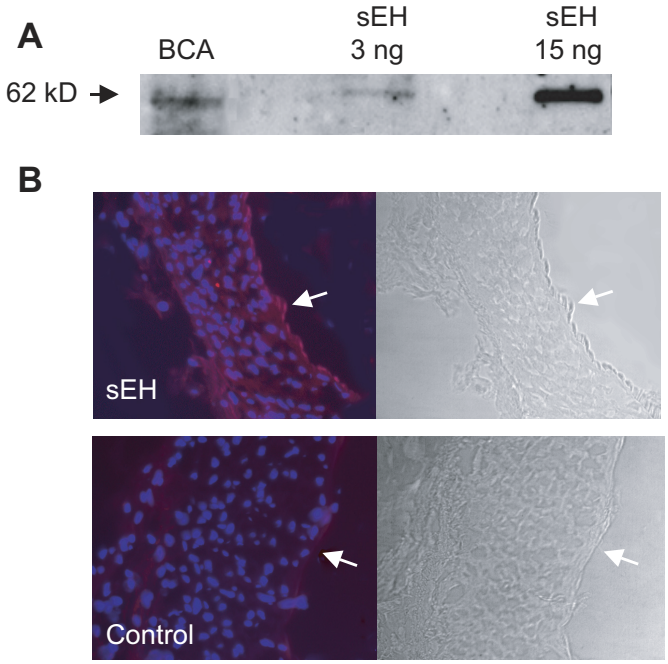


Fig. 1. Immunological characterization of sEH expression in BCAs. A, Western blot analysis of BCA lysates (25 μg) and recombinant human sEH (3 and 15 ng). B, fluorescent images and corresponding Nomarski images (20×) of BCA sections stained for sEH (red). Nuclei are stained with DAPI (blue). Sections were prepared with (top) or without the sEH antibody (control, bottom). Arrows indicate location of the endothelial cell layer.

Effect of AUDA on the Inhibition of EET-Induced Relaxations by 14,15-EE5ZE. The effect of 14,15-EE5ZE was tested on EET-induced relaxation in the presence and absence of AUDA to determine the influence of sEH on the antagonist activity of 14,15-EE5ZE. In the U46619 preconstricted arteries, 11,12-EET and 14,15-EET caused concentration-dependent relaxation of arterial rings with maximum relaxation of 86.20 ± 3.73 and 74 ± 5.3%, respectively (Fig. 3). Pretreatment of arteries with 14,15-EE5ZE (10 μM) caused significant inhibition ($P < 0.001$) of 11,12-EET and 14,15-EET-induced relaxations. With 14,15-EE5ZE, the maximal relaxations to 11,12-EET and 14,15-EET were reduced to 59.35 ± 5.5 and 21.45 ± 6.8%, respectively. Pretreatment with AUDA (1 μM) alone had no significant effect on 11,12-EET and 14,15-EET-induced relaxations. 14,15-EET-induced relaxations were inhibited to a similar extent by 14,15-EE5ZE in the presence and absence of AUDA (Fig. 3B). In contrast, the inhibitory effect of 14,15-EE5ZE on 11,12-EET-induced relaxation was significantly increased ($P < 0.01$) in the presence of AUDA, with maximum relaxation reduced from 59.35 ± 5.5% without AUDA to 43.50 ± 4.9% with AUDA. These data indicate that 14,15-EE5ZE is a better antagonist of 11,12-EET if its metabolism to 14,15-DHE5ZE is inhibited and suggests that 14,15-EE5ZE and its metabolite 14,15-DHE5ZE have different effects on the relaxations to 14,15-EET and 11,12-EET.

Effect of 14,15-DHE5ZE on EET-Induced Relaxations. In previous studies, 14,15-EE5ZE inhibited the relaxations to the four EET regioisomers; however, inhibition of 14,15-EET was greater than that of 11,12-EET, 8,9-EET, or 5,6-EET. To further examine whether the conversion of 14,15-EE5ZE to 14,15-DHE5ZE affects its antagonist activity, the effect of 14,15-DHE5ZE on the relaxations to the EET regioisomers was determined. In the U46619-preconstricted arteries, 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET caused concentration-dependent relaxations (Fig. 4). The extent of relaxation and potency of the EETs were similar. The maximum relaxation to the EETs ranged from 82 to 93% (Fig. 4). The relaxant effects of 5,6-EET, 8,9-EET, and 11,12-EET were not changed in the presence of 14,15-DHE5ZE (10 μM; Fig. 4). However, 14,15-DHE5ZE pretreatment significantly inhibited ($P < 0.001$) 14,15-EET-induced relaxation (Fig. 4D). The maximum relaxation to 14,15-EET was decreased from 83.4 ± 4.5 to 36.1 ± 9.0% after 14,15-DHE5ZE pretreatment. These findings explain the ability of AUDA to enhance the antagonist activity of 14,15-EE5ZE against 11,12-EET relaxations. The antagonist specificity of 14,15-DHE5ZE is clearly different from its parent compound 14,15-EE5ZE (Gauthier et al., 2002a).

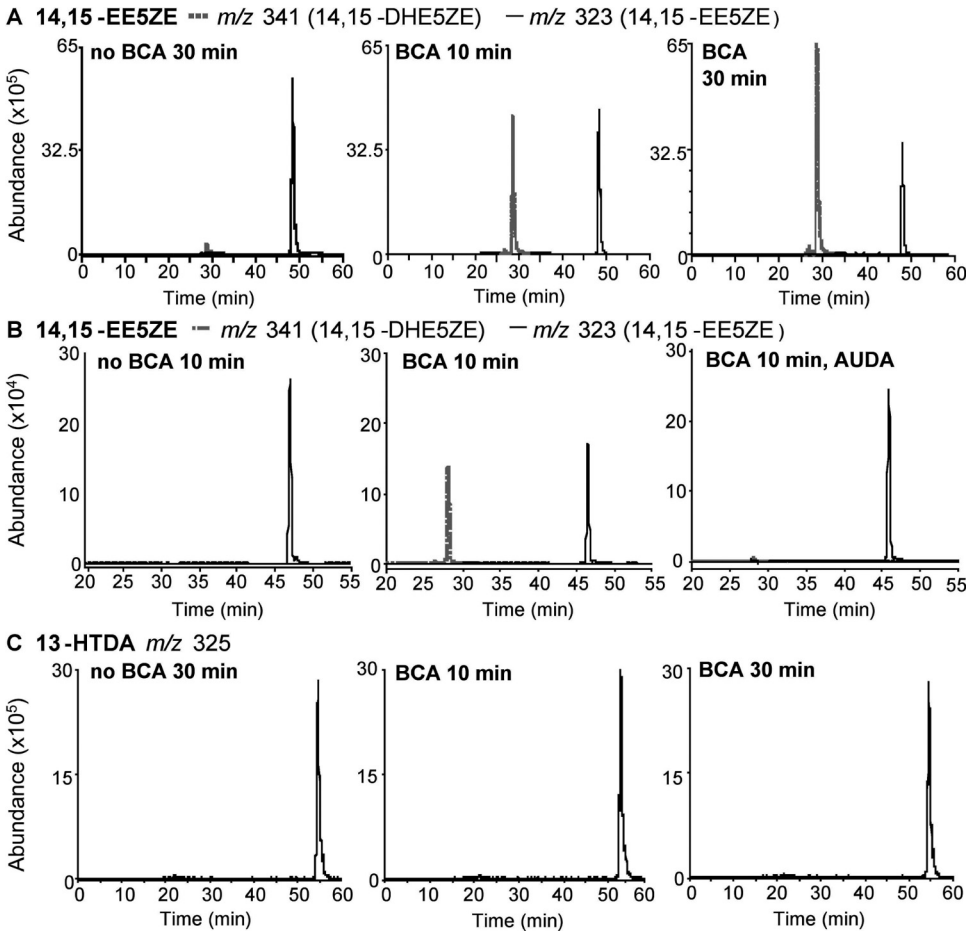


Fig. 2. A and B, metabolism of 14,15-EE5ZE (A) and effect of sEH inhibitor (AUDA) on 14,15-EE5ZE metabolism (B) in BCAs. 14,15-EE5ZE was incubated for 10 and 30 min in the presence and 30 min in the absence of BCA. Arteries were preincubated for 30 min with AUDA (1 μM) before incubation for 10 min with 14,15-EE5ZE (B). C, 13-HTDA was incubated for 10 and 30 min in the presence and absence of BCA. Medium was extracted and analyzed by LC/ESI-MS. sEH activity was expressed as the conversion of 14,15-EE5ZE to 14,15-DHE5ZE.

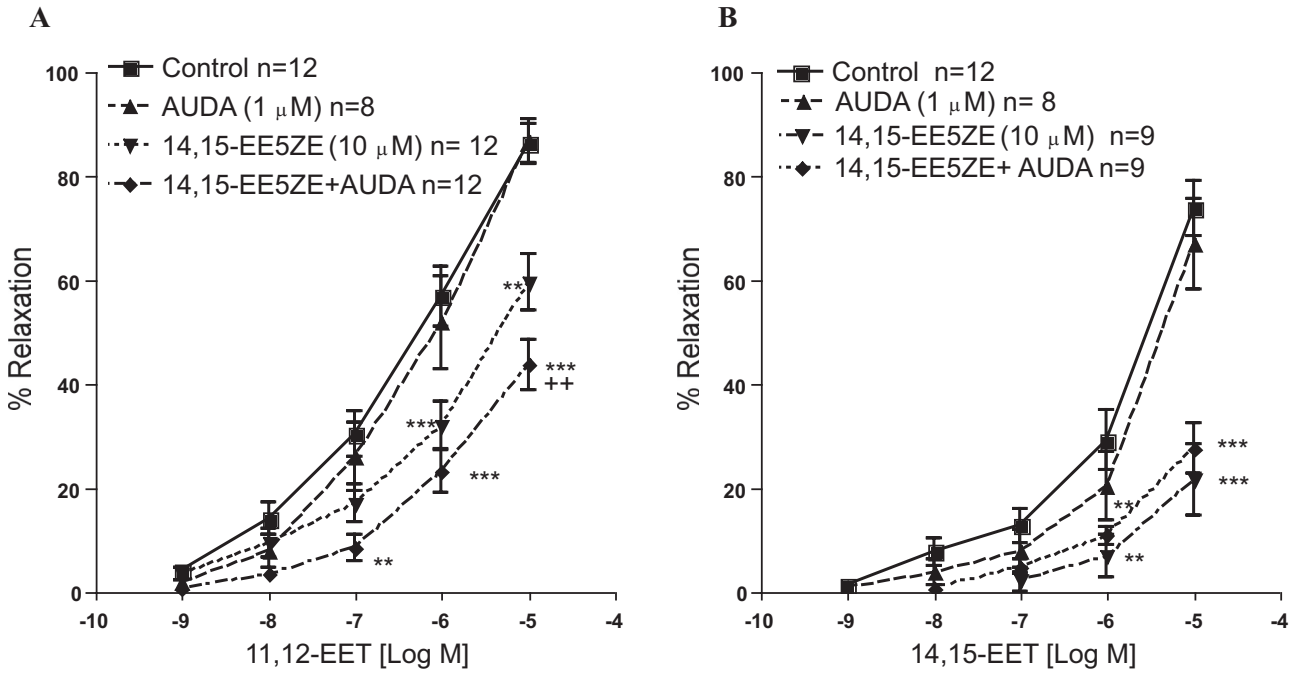


Fig. 3. Effect of 14,15-EE5ZE and AUDA on 11,12-EET-induced (A) and 14,15-EET-induced (B) relaxations in BCAs. Arterial rings were preincubated for 30 min with AUDA (1 μM), and 14,15-EE5ZE was administered before preconstriction with U46619 (20 nM). The effect of cumulative concentrations of EETs was determined. Values are mean ± S.E.M. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$ (significant difference compared with control). ++, $P < 0.01$ (significant difference compared with 14,15-EE5ZE).

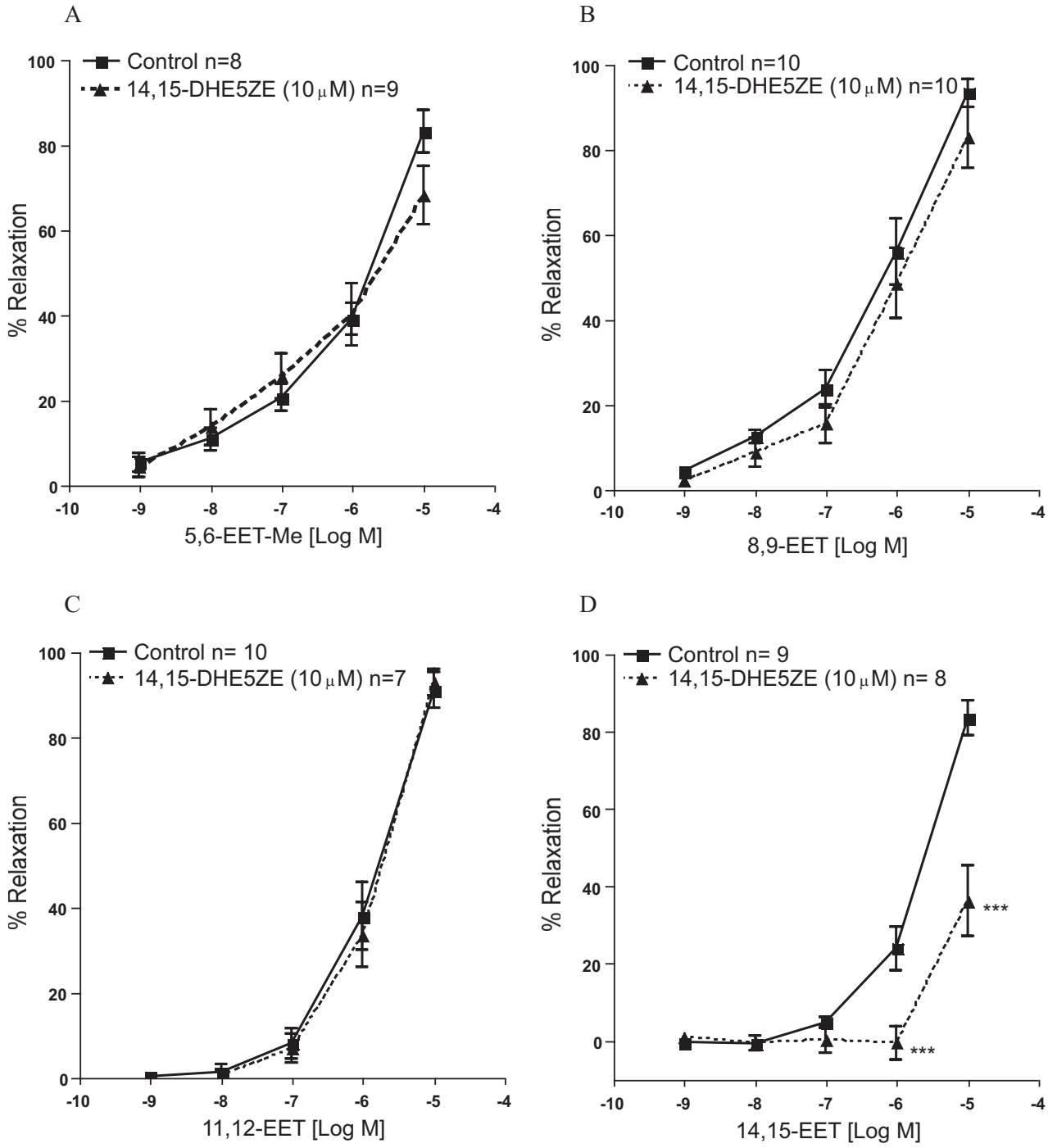


Fig. 4. Effect of 14,15-DHE5ZE on EET-induced relaxations in BCAs. Arterial rings were incubated with 14,15-DHE5ZE before preconstriction with U46619 (20 nM). Cumulative concentrations of EETs were tested. Values are mean ± S.E.M. ***, $P < 0.001$ (significantly different from control).

Effect of 14,15-DHE5ZE on AA and SNP Relaxations. AA caused concentration-dependent relaxations in the presence of indomethacin (Fig. 5A). These relaxations were reduced by 14,15-DHE5ZE (maximal relaxations: 81.0 ± 5.6% for control and 44.0 ± 7.8% with 14,15-DHE5ZE). SNP also caused concentration-dependent relaxations of coronary arterial rings. These relaxations were not altered by pretreatment with 14,15-DHE5ZE (10 μM; Fig. 5B). Similar results were obtained with 14,15-EE5ZE.

Agonist and Antagonist Effects of 14,15-EE5ZE Ether Analogs in BCAs. To avert metabolism by sEH, analogs of 14,15-EE5ZE with an ether rather than an epoxide were synthesized and tested for agonist and antagonist activity (Fig. 6A). Substitution of ether at C-13, C-14, and C-15 of 14,15-EE5ZE produced 13-HTDA, 14-HTDA, and 15-PPDA, respectively. In addition, substituting a -OH group at C-20 and/or moving the double bond from Δ5 to Δ8 of 14-HTDA produced 20-hydroxy-14-HTDA and 14-HTD-8Z-A. The agonist activities of these compounds were compared with those of 14,15-EET (Fig. 6B). 14,15-EET produced concentration-dependent relaxations with maximum relaxations of 81 ± 4.5%. The ether analogs exhibited a range of agonist proper-

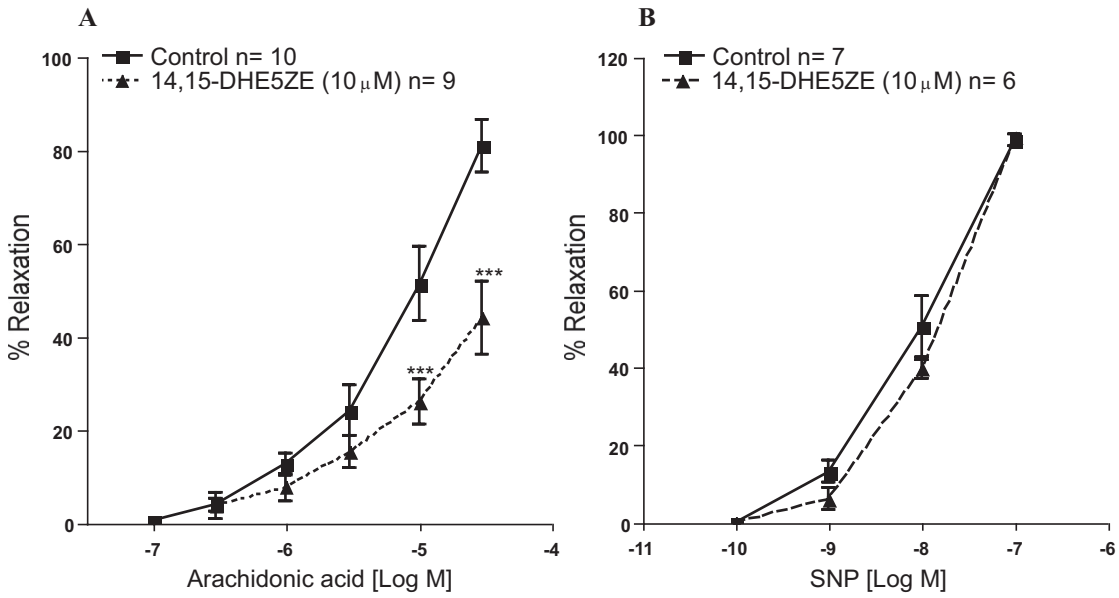


Fig. 5. Effect of 14,15-DHE5ZE on AA (A) and SNP-induced (B) relaxation in BCAs. For AA, arterial rings were pretreated with indomethacin (10 μM) before their preconstriction with thromboxane mimetic, U46619 (20 nM). Cumulative concentrations of AA or SNP were added. ***, $P < 0.001$ compared with control.

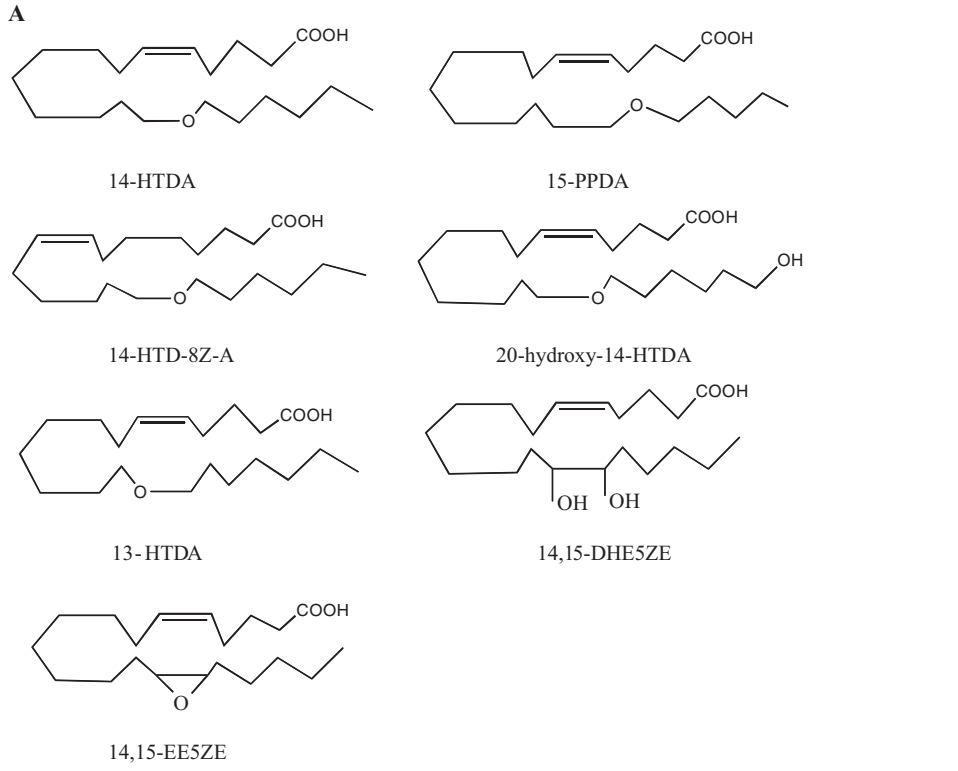
ties. The substitution of an ether for the epoxide group in the 14 (14-HTDA) or 15 (15-PPDA) position gave greater agonist activity than occurs with 14,15-EE5ZE (26% relaxation at 10 μM) (Fig. 6, B and C). Like 14,15-EE5ZE, 13-HTDA was a weak agonist, producing maximum relaxation of 23.5 ± 6.3%. Modification in the structure of 14-HDTA, such as moving the olefin from the Δ5 to Δ8 position (14-HTD-8Z-A), resulted in a loss of agonist activity (Fig. 6C). However, 20-hydroxy-14-HTDA was a better agonist than 14-HTDA and was equal to 14,15-EET, with maximum average relaxation of 75.8 ± 4.8%. Thus, whereas an ether can substitute for an epoxide, the structure-activity relationships established for 14,15-EET are markedly changed with the ether analogs. For example, 14-HTD-8Z-A is much less active than 14,15-EET, whereas 14,15-EE8ZE is equal in activity to 14,15-EET (Falck et al., 2003). The overall order of agonist potency and efficacy of the ether analogs is 20-hydroxy-14-HTDA > 14-HTDA = 15-PPDA > 14-HTD-8Z-A > 13-HTDA.

13-HTDA, an ether analog with weak agonist activity, was tested for antagonist activity. In the U46619-preconstricted arteries, 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET caused concentration-dependent relaxations with maximum average relaxation of 67.5 ± 5.3, 82.0 ± 3.6, 79.6 ± 4.0, and 78.2 ± 5.7%, respectively (Fig. 7). Previous studies have revealed that 5,6-EET is unstable at physiological buffer and is hydrolyzed to 5,6-DHET. The methyl ester analog of 5,6-EET (5,6-EET-Me) is more stable, so it was used as an alternative 5,6-EET agonist (VanderNoot and VanRollins, 2002; Yang et al., 2005). Unlike other EET regioisomers, 5,6-EET is a substrate for cyclooxygenase that metabolizes it to vasodilatory 5,6-epoxy-prostaglandins (Oliw, 1984; Carroll et al., 1993). This effect was substantiated in the present study when pretreatment of arteries with indomethacin (10 μM), a cyclooxygenase inhibitor, caused marked reduction of 5,6-EET-Me–induced relaxation (data not shown) (Yang et al., 2005). 13-HTDA pretreatment (10 μM) caused significant inhibition of 8,9-EET-, 11,12-EET-, and 14,15-EET-induced relaxations. The maximum relaxations ranged from 57 to 62% in the presence of 13-HTDA. 5,6-EET-Me-induced relaxations were unchanged in the presence of 13-HTDA. These observations indicate that changes in the 14,15-epoxy group of 14,15-EE5ZE modify the antagonist properties of the compound. 13-HTDA (10 μM) is not as effective as 14,15-EE5ZE (10 μM) in inhibiting EET-induced relaxations, despite its greater stability to sEH metabolism.

Discussion

EETs are P450 metabolites of AA that hyperpolarize and relax vascular smooth muscle by activating BK_{Ca} channels and causing membrane hyperpolarization (Campbell et al., 1996; Li and Campbell, 1997; Fisslthaler et al., 1999). The four EET regioisomers, 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET, are equipotent in relaxing coronary arterial rings. The vascular relaxant effect of EETs is blocked by the 14,15-EET analog 14,15-EE5ZE (Gauthier et al., 2002a). In coronary arteries, EETs are hydrolyzed to corresponding DHETs by sEH (Weintraub et al., 1997; Campbell et al., 2002; Spector and Norris, 2007). In previous studies, the metabolism of EET to DHETs was decreased in the presence of 14,15-EE5ZE, suggesting that 14,15-EE5ZE competes with EETs for sEH and reduces the conversion of EETs to DHETs (Gauthier et al., 2002a).

In the present study, we found that BCAs and endothelial cells express sEH and arteries convert 14,15-EE5ZE to 14,15-DHE5ZE. The metabolism of 14,15-EE5ZE was blocked completely by AUDA, a sEH inhibitor (Morisseau et al., 1999), confirming the hypothesis that 14,15-EE5ZE serves as a substrate for sEH (Gauthier et al., 2002a). 14,15-EE5ZE nonselectively inhibits the vascular relaxation to all four EET regioisomers; however, its metabolite 14,15-DHE5ZE causes specific inhibition of only 14,15-EET-induced relaxation. 14,15-DHE5ZE pretreatment had no significant effect on the other EETs. The reason for this specific inhibition is not clear. This effect was further confirmed when AUDA pretreatment significantly increased the antagonist effect of



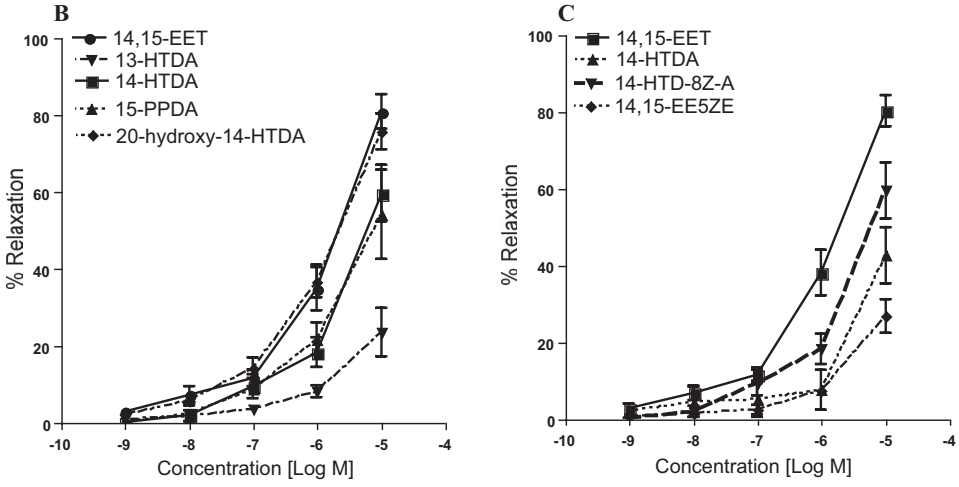


Fig. 6. Chemical structures of 14,15-EE5ZE ether analogs (A) and comparison of 13-HTDA, 14-HTDA, 15-PPDA, 20-hydroxy-14-HTDA, 14-HTD-8Z-A, 14,15-EE5ZE, and 14,15-EET-induced relaxations in BCAs (B and C). Arterial rings were preconstricted with U46619 (20 nM). Cumulative concentrations of the analogs were tested. Values are mean ± S.E.M. (n = 5–9).

14,15-EE5ZE against 11,12-EET-induced relaxations, indicating that AUDA blocked 14,15-EE5ZE metabolism by sEH. 14,15-EE5ZE inhibited 14,15-EET-induced relaxations to a similar extent in the presence and absence of AUDA. 14,15-EE5ZE-mSI, a structural analog of 14,15-EE5ZE, caused specific inhibition of 5,6-EET and 14,15-EET but was without effect on the relaxations to 11,12-EET and 8,9-EET (Gauthier et al., 2003). These observations indicate that substitution at the carbon-1 carboxyl group and hydrolysis of the 14,15-epoxy group modify the antagonist specificity of 14,15-EE5ZE. Moreover, the nonselective inhibitory effect of 14,15-EE5ZE against EETs is diminished by its metabolism by sEH in the vasculature.

We synthesized a series of sEH-resistant analogs of 14,15-EE5ZE and characterized their agonist and antagonist properties on BCA rings. These compounds differ from 14,15-EE5ZE in that the 14,15-epoxy group is replaced with 13-, 14-, and 15-ether groups (13-HTDA, 14-HTDA, and 15-PPDA). Marked agonist activity was observed with the conversion of 14,15-epoxy group of 14,15-EE5ZE to 14- and 15-ether groups. Addition of a hydroxyl group at carbon-20 enhanced the agonist activity of 14-ether analog. Changing the double bond from Δ5 to Δ8 decreased agonist activity of 14-HTDA, indicating that the Δ5 olefin and carbon-20 hydroxyl group are necessary for the full agonist activity of the 14-ether analogs. 13-HTDA was a weak agonist activity, so it was tested for antagonist. Pretreatment of arteries with 13-HTDA significantly inhibited 8,9-EET-, 11,12-EET-, and 14,15-EET-induced relaxations. 5,6-EET-induced relaxations were unchanged. These findings suggest that changing the 14,15-epoxy group of 14,15-EE5ZE to 14- and 15-ether groups results in loss of antagonist activity. Substituting the 14,15-epoxy with a 13-ether analog results in retention of antagonist activity, but the 13-ether analog was a weaker antagonist than 14,15-

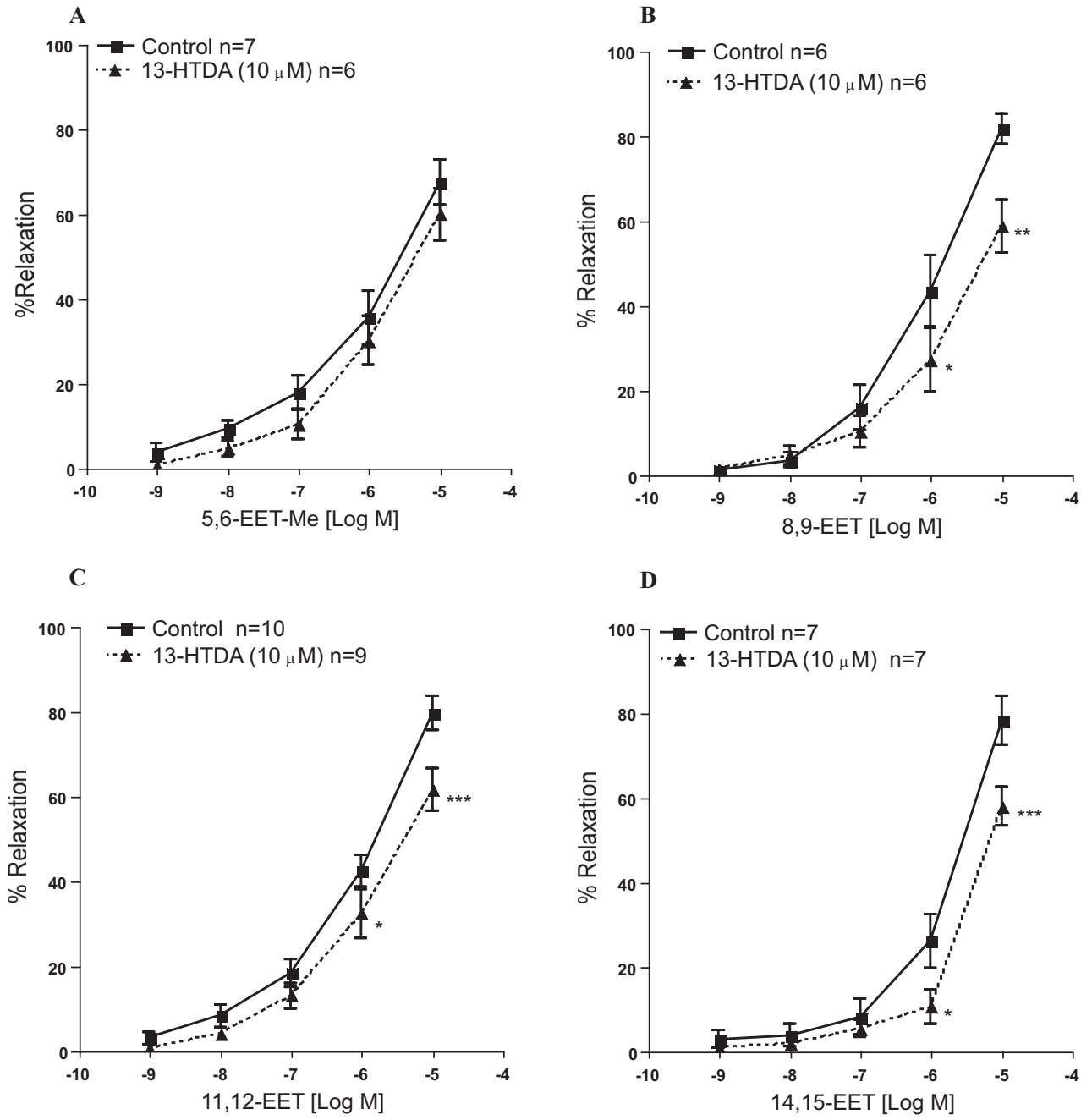


Fig. 7. Effect of 13-HTDA on EET-induced relaxations in BCAs. Arterial rings were pretreated with 13-HTDA before their preconstriction with U46619 (20 nM). Cumulative concentrations of the EETs were tested. Values are mean ± S.E.M. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$ (significant inhibition compared with control).

EE5ZE, suggesting that an epoxide group is required for maximal antagonist activity. However, mass spectrometric analysis of 14,15-EE5ZE metabolism by coronary arteries showed conversion of the epoxy group to the vicinal diol by sEH. This metabolism may limit the use of 14,15-EE5ZE as an EET antagonist. Unlike 14,15-EE5ZE, 13-HTDA was resistant to metabolism by sEH because of the lack of an epoxy group in its structure. The mass spectrometric analysis of 13-HTDA metabolism by coronary arteries showed no conversion to other products. 13-HTDA is a metabolically stable EET antagonist. Further structural modification of this compound may furnish a potent, stable EET antagonist.

In summary, the results from this study show that 14,15-EE5ZE is metabolized to 14,15-DHE5ZE by sEH in the BCAs. 14,15-DHE5ZE specifically inhibits 14,15-EET-induced relaxation. The EET regioisomer-specific inhibition by 14,15-DHE5ZE raises the possibility that the four EET regioisomers may have different receptors or binding sites in the vasculature. Efforts are under way to identify EET receptor(s) (Wong et al., 1993; Yang et al., 2007, 2008; Chen et al., 2009). The regioisomer-specific EET antagonists such as 14,15-DHE5ZE will be valuable in characterizing the functional role of individual EETs in biological responses.

The ether analogs of 14,15-EE5ZE are not metabolized by sEH so may be helpful in understanding the functional role of endogenous EETs in conditions where sEH expression is

up-regulated (Ai et al., 2007). 13-HTDA may serve as a useful alternative for 14,15-EE5ZE; however, further modifications to its structure are required to achieve better antagonist activity. These analogs form the basis for the development of metabolically stable EET antagonist analogs and EET mimetics that may be used to treat cardiovascular disorders and inflammation.

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Authorship Contributions

Participated in research design: Bukhari, Gauthier, Falck, and Campbell.

Conducted experiments: Bukhari, Gauthier, Jagadeesh, and Sangras.

Contributed new reagents or analytic tools: Jagadeesh, Sangras, and Falck.

Performed data analysis: Bukhari.

Wrote or contributed to the writing of the manuscript: Bukhari, Gauthier, Falck, and Campbell.

Other: Falck and Campbell acquired funding for the research.

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